

**Milk adulteration with acidified rennet whey: a limitation for the
caseinomacropeptide detection by high-performance liquid
chromatography**

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ABSTRACT

BACKGROUND: High-performance liquid chromatography (HPLC) is widely employed to determine the caseinomacropeptide (CMP) index and detect milk tampering with rennet whey. Prior to HPLC analysis, CMP is subject to a trichloroacetic acid (TCA) isolation causing further soluble proteins in the sample to precipitate. On this basis, we wanted to estimate if rennet whey acidification could adversely affect the HPLC sensitivity to detect this peptide. **RESULTS:** As hypothesized, the CMP index from milk added with acidified rennet whey was, on average, half as less as that quantified from milk added with rennet whey. Moreover, *quantum satis* of acidified whey added into milk enough to evidence a HPLC CMP > 30 mg L⁻¹ was 94% greater than that required for this threshold to be reached with rennet whey. **CONCLUSION:** Milk tampering with acidified rennet whey may limit the analytical sensitivity of the reversed-phase HPLC employed on the screening of CMP, and ultimately, disguise the fraudulent addition of whey to milk.

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KEYWORDS

Milk adulteration, fraud, sour whey, glycomacropeptide.

INTRODUCTION

The raw milk tampering with rennet/cheese whey from curd cheese making is a food fraud of concern to dairy processors and food inspection services of developing countries¹⁻², undermining dairy markets and infringing basic consumers' rights.³ However, milk and cheese whey share common matrices, suggesting that changes on physicochemical characteristics of milk added with cheese whey are not evident,⁴ and that the detection of such an alteration by quality control testing routinely employed at reception of raw milk in the dairy plant is even challenging.³ Depending on the initial content of fat in raw milk, it is still possible to add up to 10% of whey without extrapolate any compositional parameter.⁵ This led to the development of HPLC-based tools for the *a posteriori* quantitation of the cheese whey peptide marker in finished dairy products, the caseinomacropeptide (CMP).² The procedure requires a selective precipitation of interfering proteins from the sample⁶, usually by trichloroacetic acid (TCA) at 8 ml dL⁻¹, prior to the analysis. Ideally, CMP peak heights ≤ 30 ml dL⁻¹ indicate that the raw milk employed was authentic.⁴ Conversely, higher peaks may denote that the raw milk was likely tampered with whey. However, as one can expect, an insufficient removal of whey proteins may result whether TCA at low concentrations is used. On the other hand, extensive precipitation of proteins - and ultimately of CMP - may occur if a TCA at high concentrations is employed.⁶ Bearing that in mind, we hypothesized that an over acidification of rennet whey could adversely influence the analytical sensitivity of HPLC, affecting the accurate estimate of the rennet whey added to raw milk. Therefore, from an

economic standpoint, a deliberate addition of an acidifier to curd cheese whey prior to its addition into milk could represent an attractive means of milk fraud through a malicious perspective. For that reason, our study was undertaken to contribute for the understanding on the analytical sensitivity of the HPLC method employed to detect CMP from samples of untampered, raw milk, altered with rennet whey, altered with sour whey, and from milk tampered with acidified rennet whey.

MATERIAL AND METHODS

A batch of raw bovine milk (40 L) from the farm bulk tank (4°C) within 24 hours from milking was used to build the standard curves for CMP HPLC and fluidized whey preparation. The percentages of fat, protein, lactose and solids were measured by infrared.⁷ The somatic cell count (SCC)⁸ and total bacterial count (TBC)⁹ were measured by flow cytometry. Standard curves for HPLC CMP were prepared in a deionized water solution 1:10 (w/w) of 90% CMP standard (Dinâmica®, Brazil).¹⁰

Raw milk was split and three different types of whey were produced: rennet whey, sour whey and acidified rennet whey. Rennet whey was obtained by adding enzymatic coagulant (Estrella®, Chr-Hansen A/S, Valinhos, Brazil) at raw milk (0.7 mL L⁻¹ of milk). The mixtures of milk added with coagulant were stirred for 3 minutes and then allowed to set for 30 min. Sour whey was obtained by adding lactic acid (85 mL dL⁻¹) at raw milk (1.6 mL L⁻¹ of milk). The mixtures were stirred for 3 minutes and then allowed to set for 30 min. At the end of set, the milk coagula were cut and curds were agitated gently with no heat for 5 min. Next, the curd plus whey was heated from 30 to 70°C during 07 min and the whey was removed from the vat with a stainless sieve. Finally, the resulting whey was heated at 80°C for 15 min and frozen at -18°C until chromatographic analyses. The acidified rennet whey was obtained by adding lactic acid (85 mL dL⁻¹) at rennet cheese whey (0.23 g dL⁻¹) to achieve a final pH of 5.95 (equals to acid cheese whey). The three types of whey were subject to physicochemical analyses as previously described.^{7,8,9,10}

The three types of whey were also assessed for pH (Tec 5[®], Tecnal, Piracicaba, Brazil) titratable acidity and freezing point (MK 540 FLEX[®], ITR, Esteio, Brazil).

In order to simulate the adulterated milk, samples were prepared with the addition of each type of adulterant (rennet, sour and acidified renet) at a ratio of 0, 1, 5, 10, 25 and 50 ml dL⁻¹.

Detection of CMP was carried out on the altered milk samples using reversed-phase HPLC. Analyses were performed in duplicate. Isolation of CMP from both the unaltered raw milk and adulterated milk samples was performed as follows: ten milliliters of the sample were added with 5 mL of 24 ml dL⁻¹ TCA and allowed to precipitate (60 min).¹⁰ The precipitate was removed (Whatman[®] filter paper N° 5, Sigma-Aldrich, St. Louis, USA) and the resulting filtrate was submitted to chromatography analysis.¹¹

RESULTS AND DISCUSSION

Somatic cell count and physicochemical results of raw milk and tampered milk used in the experiment are show in Table 1. The level of endogenous CMP in raw milk used in this work was 2.55 mg L⁻¹, results within the range found by Motta et al.⁴ and within the Brazilian statutory¹⁰ tolerance level, *i.e.* 30 mg L⁻¹. About compositional parameters, our results showed that is possible to add up to 10% of any type of whey without extrapolate the specified limits for an authentic milk.

However, CMP levels were progressively increased by adding renet whey to raw milk (Figure 1). The tolerance level and the detection limit of the method taken into consideration, CMP could be accurately detected in samples with renet whey at a ratio of 5 ml dL⁻¹, 10 ml dL⁻¹, 25 ml dL⁻¹ and 50 ml dL⁻¹ (Figure 1). As CMP concentration in renet whey ranges from 1.2 to 1.5 g L⁻¹, thus 30 mg L⁻¹ is equivalent to 2 - 4 ml dL⁻¹ of renet whey in fluid milk.⁴

As expected, the addition of sour whey did not increase CMP concentration. Even raw milk added with 50 ml dL⁻¹ sour whey did not exceed the tolerance level of 30

mg L⁻¹, reaching maximum values at about 7.3 mg L⁻¹ (Figure 1). Non-detectable peaks were also reported¹² when acidic whey was subject to reversed-phase HPLC analysis. This type of milk adulteration, *i.e.* addition of acidic whey, is better identified by methods based on the compositional difference between whey and milk (non-CMP-based methods), such as the determination of casein-bound phosphorous or casein/whey proteins ratio by SDS-PAGE.¹³

As hypothesized, CMP peak heights did not increase proportionally with the addition of acidified rennet whey to raw milk as it did with the addition of rennet whey into raw milk. Heights recorded over the set of samples with acidified whey were on average twice as low (~52%) as those observed among samples with rennet whey (Figure 1).

Findings reported here suggest that CMP from raw milk tampered with up to 3.5 ml dL⁻¹ of acidified rennet whey might go undetected by the HPLC. Quantity-wise, the volume of acidified rennet whey in raw milk needed to make up a CMP concentration that exceeds the statutory limit of 30 mg L⁻¹ is 94% greater than that required to surpass that threshold with the addition of whey to milk, as extrapolated by least square fitting of scattering plots found in the CMP quantitation herein.

Similarly, underestimation of CMP also seemed likely to occur from milk samples added with acidified rennet whey acidified with lactic acid (85 ml dL⁻¹), which could be a consequence of the differences on precipitation susceptibility found among the non-glycosylated fractions of CMP and the type of acid employed for its isolation.¹² Owing to these, our results suggest that 52% of CMP might have undergone precipitation as when raw milk was added with rennet whey acidified with 0.23 g dL⁻¹ of lactic acid added (85 ml dL⁻¹ and a final concentration of lactic acid in whey of 0,17 g L⁻¹), seemingly leading its screening by the HPLC method, as referred by a CMP threshold limit of 30 mg L⁻¹, to go undetected.

Yet, this framework must represent a matter of some concern to dairy industries and food safety authorities as it may become a means to disguise whey addition to milk. Finally, the results provided here may indicate that any condition - inadvertent or deliberate -, leading to the acidification of whey, is also likely to influence the analytical sensitivity of HPLC towards an accurate detection of CMP, which ultimately, may breach consumers' rights to authentic purchasing of dairy products and unbalance their fair trading between market players. Therefore, findings presented here suggest that the precipitation behavior of CMP in acidic solutions must be taken into consideration for a precise assessment of milk adulteration with cheese whey by HPLC.

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TABLES

Table 1. Physicochemical composition and somatic cell count of raw milk, rennet whey, acidified rennet whey and sour whey used throughout the experiment.

Parameters	Raw milk	Rennet whey	Acidified	
			rennet whey	Sour whey
pH	6.72	6.45	5.95	5.95
Titrateable acidity (g dL ⁻¹)	0.16	0.16	0.18	0.18
Freezing point (° H)	-0.548	-0.545	-0.602	-0.831
Fat (ml dL ⁻¹)	3.43	0.56	0.55	0.5
Protein (ml dL ⁻¹)	3.15	1.00	1.02	1.13
Nonprotein nitrogen (mg dL ⁻¹)	13.70	8.04	8.11	22.01
Casein (ml dL ⁻¹)	2.51	1.74	1.71	1.81
Lactose (ml dL ⁻¹)	4.73	4.95	4.91	5.17
Total Solids (ml dL ⁻¹)	12.25	7.27	7.23	7.68
Somatic cell (10 ⁵ cells mL ⁻¹)	1.51	0.12	0.12	0.70

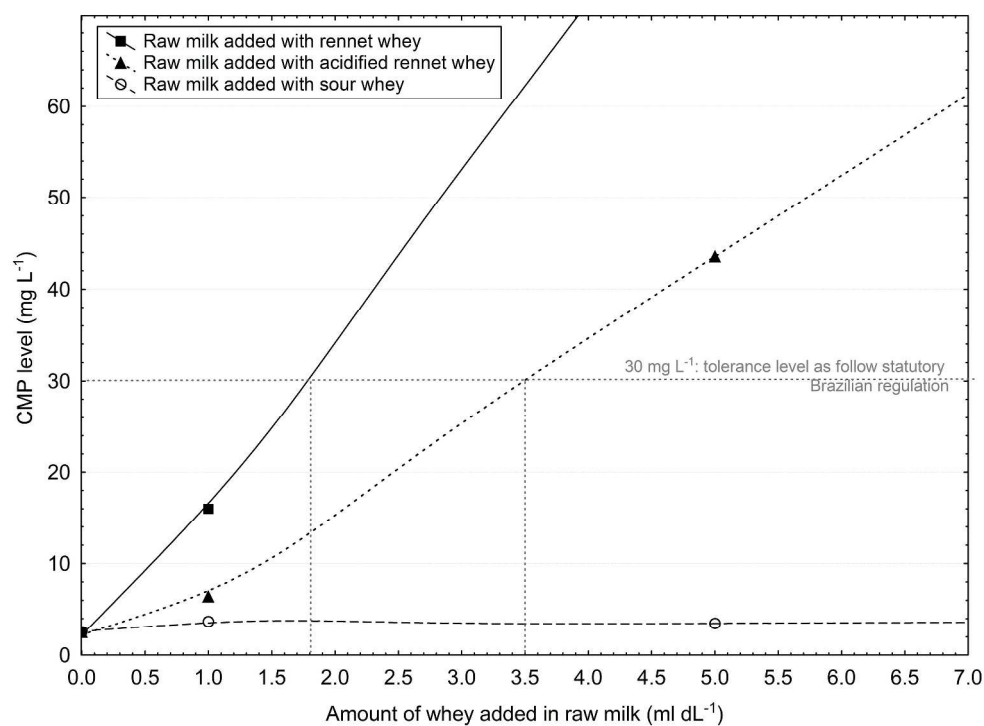


Figure 1. Least square fitting of scattering plots found in CMP quantitation of raw milk samples added with sour whey, rennet whey and acidified rennet whey.